EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1393

VISUAL CHARACTERIZATION OF VX DROPLETS ON PLANT FOLIAGE

Michael Simini
Ronald T. Checkai
Mark V. Haley

RESEARCH AND TECHNOLOGY DIRECTORATE

July 2016

Approved for public release; distribution is unlimited.

U.S. ARMY RDECOM

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-07-2016	Final	May 2014 – Sep 2015

4. TITLE AND SUBTITLE
Visual Characterization of VX Droplets on Plant Foliage

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Simini, Michael; Checkai, Ronald T.; and Haley, Mark V.

5d. PROJECT NUMBER
WBS R.0013813.81.4

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ECBC, ATTN: RDCB-DRT-M, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1393

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency; 8725 John J. Kingman Road, MSC 6201; Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)
DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release, distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT-LIMIT 200 WORDS
A method was developed for maintaining healthy plants in a chemical surety hood and for disseminating, observing, and evaluating VX [*O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothioate] droplet spread, absorption, and appearance on leaves. In separate experiments, 1 or 3 µL droplets were dispensed onto plant leaves using a high-precision syringe. Digital photographs of the droplets were taken at intervals, saved, and analyzed. Each image was electronically traced and converted to surface area (SA) using Image-Pro Insight software (Media Cybernetics; Rockville, MD). Droplets on foliage initially appeared as transparent areas and spread with time. After 1 h, the 1 and 3 µL droplets ceased to spread, the VX appeared fully absorbed into leaf tissue, and the leaf tissue within the spread area appeared dry and darkened. After 24 h, the affected leaf tissue appeared light tan and necrotic. Mean SAs of the affected tissues for 1 µL droplets were 132, 192, 163, 135, and 142 mm^2 at 0.017, 0.05, 1, 4, and 24 h, respectively, and those for 3 µL droplets were 166, 301, 303, 278, and 274 mm^2 at 0.05, 1, 4, 24, and 48 h, respectively. Using these results can accelerate field identification and characterization of VX to more effectively protect Warfighters.

15. SUBJECT TERMS
Chemical warfare agent (CWA) — *O*-Ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothioate (VX)
Plants — Foliage — *Echinochloa crus-galli* — Barnyard grass

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)* (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized under project no. WBS R.0013813.81.4 and supported by the Defense Threat Reduction Agency (DTRA). This work was started in May 2014 and completed in September 2015.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

Acknowledgments

The authors would like to thank Dr. Terrance G. D'Onofrio (U.S. Army Edgewood Chemical Biological Center [ECBC]; Aberdeen Proving Ground, MD) for providing access to Image-Pro Insight imaging software (Media Cybernetics, Inc.; Rockville, MD) and instructions regarding applications, and Dr. David J. McGarvey (ECBC) for conducting VX purity determinations using NMR. The authors would also like to thank Dr. Bruce King (ECBC) and Dr. Morgan Minyard (DTRA; Fort Belvoir, VA) for consultation throughout the duration of the project.

Blank

CONTENTS

FIGURES

VISUAL CHARACTERIZATION OF VX DROPLETS ON PLANT FOLIAGE

1. INTRODUCTION

Developing advanced defensive capabilities against chemical warfare agents (CWAs) requires greater understanding of the underlying principles that affect CWA persistence, partitioning, contact transfer (exposure), and transport in the environment. Because some CWAs have relatively low vapor pressure, contact with contaminated surfaces is considered to be a primary route of exposure for the Warfighter. Toxicological investigations have shown that direct exposure of organisms to various CWA-contaminated surfaces can present a contact hazard (D'Onofrio, 2013). Currently, little experimental data exist that describe CWA–plant interactions. A standardized protocol with intact living plants was needed to maintain plant physiological responses and obtain results applicable to CWA-contaminated battlefields. Furthermore, without a more-complete understanding of these interactions, it was difficult to predict the persistence of the potential hazard posed by CWAs in a natural environment, much less that arising from CWA interaction with plants. Methods were needed for growing and maintaining healthy plants in a chemical surety hood to observe and evaluate the appearance and extent of spread of droplets that were disseminated onto the foliage of intact living plants.

2. BACKGROUND

Persistence, environmental fate, and partitioning of CWAs into abiotic (e.g., air, water, soil, and sediment) and biotic (e.g., human) components in natural environments, after dissemination onto foliar surfaces in the field, can be influenced by the indigenous vegetation. The organophosphorus (OP) CWA, VX [*O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothioate] is, by any route of exposure to humans, one of the most potent of the known nerve agents. When compared with the G agents, VX is more toxic, more stable, more resistant to detoxification, less volatile, more efficient at skin penetration, and more environmentally persistent (Munroe et al., 1994). Because of these characteristics, VX is more effective as a skin penetrant and lethal contact agent than as an inhalation threat, although inhalation is a viable concern due to the toxicity and persistence of VX (Munroe et al., 1994; Reutter, 1999). However, all nerve agents, but especially VX, may persist in common building materials and on agricultural crops, which presents dermal and inhalation exposure hazards (Watson et al., 1989). Therefore, the characterization of visual signs and symptoms, fate, and persistence of VX that has been disseminated on indigenous vegetation is important for the estimation of risk to Warfighters on the battlefield and first responders in the event of a hostile release. Because VX can persist on leaf surfaces in an undegraded form, animals grazing on any contaminated vegetation can ingest VX. Clinical signs of toxicity persisted for at least 3 weeks in sheep that were accidentally exposed to VX-contaminated vegetation (Van Kampen et al., 1969). Up to 2 months after the VX release, slight acetylcholinesterase depression was reported in a group of newly introduced sheep that were grazing in the same area (Van Kampen et al., 1970).

Our overall goals while investigating CWA–plant interactions included developing and validating methods to assess the fate, persistence, and contact transfer potential of CWAs on intact plant leaves relative to Warfighter exposure. In this study, we examined the spread of VX droplets disseminated onto living grass foliage and characterized the pattern of injury on the surface of leaves as a function of the time of exposure after dissemination. We developed and implemented innovative methods to study these parameters on living intact plants that were exposed to CWA in chemical surety hoods. Subsequent related technical reports will address the persistence, fate, and contact transfer of VX droplets on foliage. Results from this study and ongoing studies of CWA persistence and contact transfer with plant foliage, combined with those of the previous soil studies, will provide standardized protocols for predicting the extent and duration of exposure hazards resulting from the dissemination of CWAs onto plant foliage and soils.

The successful outcome of this research will provide critical information for battlefield commanders on the nature of visually detected injury to plants from exposure to VX. This information will aid timely deployment of detection technologies in the field. It will advance scientific knowledge and understanding of selected biologically mediated processes that are altered by the presence of CWAs in the natural environment. The predictive capabilities and scientific knowledge obtained in this research will lead to enhanced force protection. Results from this research on VX–plant interactions can accelerate field characterization to more effectively protect Warfighters and aid efforts by the Department of Homeland Defense, Environmental Protection Agency, and others who are involved in mitigating the environmental release of CWAs.

3. MATERIALS AND METHODS

3.1 Chemicals

All studies described herein used the CWA VX at 93% purity, Chemical Agent Standard Analytical Reference Material grade, Chemical Abstracts Service (CAS) no. 50782-69-9, which was stabilized with 5% by weight diisopropylcarbodiimide (CAS no. 693-13-0; Sigma-Aldrich Company; St. Louis, MO). Reagent-grade isopropyl alcohol (CAS no. 67-63-0) was used as an extractant. Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Company; Marysville, OH) fertilizer (24% total nitrogen [calculated as N], 8% available phosphate [calculated as P_2O_5], 16% soluble potash [calculated as K_2O], 0.02% boron, 0.07% copper [water soluble], 0.15% iron [chelated], 0.05% manganese [chelated], 0.0005% molybdenum, 0.06% zinc [water soluble], and 1.14% ethylenediaminetetraacetic acid chelating agent) was used to prepare dilute phytonutrient solution (530 mg/L) with ASTM Type I water (18 MΩ cm) (ASTM International, 2004).

3.2 Plant Selection, Establishment, and Culture Methods

A literature search was performed to select candidate plant species for use in CWA–plant interaction studies. Grass is the most prevalent type of higher plant worldwide. The

grass species *Echinochloa crus-galli* (L.[*]) P. Beauv.[†] (a common name for this species is barnyard grass) was chosen for these studies because it is one of the most widely distributed natural grass species worldwide, is tolerant of both dry and wet natural habitats, is used as forage for grazing animals, and functions well as both food and habitat for wildlife (USDA NRCS, 2016). This species is also one of the most-important weed species in many crop systems (especially in rice) worldwide, and in some parts of the world, *E. crus-galli* is cultivated as a crop for human consumption (CABI, 2016).

Twenty seeds of *E. crus-galli* (lot no. PM11452Q, 2014; Prairie View Nursery; Winona, MN) were sown in potting mix (Miracle Gro Moisture Control potting mix; Scotts) and hydrated with ASTM Type I water. Germination (80 to 95%) time averaged 5 to 7 days. The bottoms of 100 mm (4 in.) diameter plant-growth containers (flower pots) were lined with two pieces of absorbent paper, then filled with 170 g (77.1 g dry mass) of potting mix. After 7 days, individual *E. crus-galli* seedlings were transplanted into these prepared flower pots. It was experimentally determined that each pot required 48 g of water initially and regular maintenance of dilute Miracle-Gro fertilizer to sustain healthy plant growth. The mass of each potted plant, with its initial ASTM Type I water amendment, was recorded, and all pots were placed into an environmentally controlled plant-growth chamber equipped with fluorescent and incandescent lighting (model PGC-9/2; Percival Scientific; Perry, IA). To avoid the effects of nutrient deficiencies that may exist in natural soils, dilute Miracle-Gro fertilizer (530 ± 15 mg/L) was prepared and administered to the plants every 2 to 3 days to maintain the respective initial mass of each replicate plant system.

3.3 Experimental Conditions

Traditionally, culturing plants under controlled environmental conditions outside of surety hoods involves balancing the heat loads with large chilling units that are too cumbersome for most surety hoods. Because physiologically healthy living plants are required for the investigation and recording of critical parameters for the effects of CWA–plant interactions, we first determined the experimental conditions that are required for sustaining living, physiologically healthy foliage of the grass *E. crus-galli* within a non-surety, environmentally controlled growth chamber. We then applied comparable conditions within the surety hood using a light-emitting diode (LED) system of lighting to supply high-quality, photosynthetically active radiation (PAR) at 400–700 nm (McCree, 1981).

The following test conditions were maintained in the non-surety growth chamber: temperature, 22 ± 2 °C (light) and 18 ± 2 °C (dark); relative humidity (RH), 60 ± 5%; photoperiod, 16 h (light) and 8 h (dark); airflow, 1.5 mph; and canopy light intensity, 300 to 350 µmol s^{-1} m^{-2} PAR. Temperature and RH were measured with an OM-DVTH data logger (Omega Engineering, Inc.; Stamford, CT). PAR was measured periodically using an MQ-200 quantum meter equipped with an AM-310 sensor wand (Apogee Instruments; Logan, UT). Plants with two to three fully matured leaves (18 to 21 days after transplanting) were selected for experimentation and transferred to the surety hood.

[*] L. indicates that Carl Linnaeus is the authority for the species name.
[†] P. Beauv. indicates that Palisot de Beauvois was the author of this botanical name.

The surety hood was equipped with two LumiBar LED light strips (LumiGrow, Inc.; Novato, CA) supported by an adjustable light stand (Figure 1). The selected lighting system uses compact, low-heat, energy-efficient LED lights that emit radiation primarily in the PAR spectral range with sufficient intensity to sustain the growth of physiologically healthy plants. Temperature and RH were measured with an Omega OM-DVTH data logger. The temperature within the surety hood was maintained at 22 ± 2 °C, and the RH was maintained at 50 ± 10%. The average airflow through the hood was measured at 2.4 ± 0.14 kph (measured at the face of the hood using an AirData Multimeter, ADM-870C; Shortridge Instruments, Inc.; Scottsdale, AZ).

Figure 1. *E. crus-galli* plants in surety hood with plant stands, LED lights, and photographic apparatus.

3.4 Dissemination of VX Droplets onto Leaves

Plant stands were constructed to hold the flower pots in a fixed position. Each pot was placed through a hole cut in a Petri dish cover and onto a 140 mm (5.5 in.) diameter Petri dish, and it was then secured to a ring stand with an adjustable ring clamp (Figure 2). Plant leaves were laid horizontally across a ring near the top of the plant canopy and secured to the ring with lengths of clear plastic (cellulose acetate) tape that were folded in half lengthwise (thus preventing sticky contact of acrylate adhesive to the leaf surface) and placed across the leaf surface. The ends of the folded tape were then secured to the ring with additional tape, while maintaining slight pressure on the leaf (Figure 3). This method of securing individual leaves in a horizontal position prevented any possible leaf surface damage caused by tape removal and

ensured that disseminated agent droplets contacted the leaf surface at the point intended and that those locations could be easily identified for further investigation. Individual plant leaves remained secured in this horizontal position during and after dissemination of VX to prevent uncontrolled deposition of agent throughout testing.

Figure 2. *E. crus-galli* plants with plant stands in surety hood.

Figure 3. Close-up view of *E. crus-galli* leaves that have been horizontally secured to plant stand in surety hood. Lengths of clear adhesive tape, folded in half lengthwise, were placed across the leaf surface to prevent sticky contact of tape to leaf surfaces. The ends of the folded tape were then secured to the ring with additional tape to maintain slight pressure.

In separate experiments, single 1 or 3 µL VX droplets, representing the range of droplet sizes expected from CWA dissemination under field conditions (TOP, 2011), were individually dispensed onto plant leaves. The spread of the absorbed 1 µL VX droplets were digitally photographed at 0.017, 0.05, 1, 4, and 24 h, and the spread of 3 µL VX droplets were photographed at 0.05, 1, 4, 24, and 48 h. Three 3 µL droplets of ASTM Type I water were applied to separate leaves for comparison.

3.5 Imaging of Leaf to Determine Surface Area (SA) of VX Droplet Spread

A 140 mm (5.5 in.) diameter Petri dish was used to hold each leaf for photographing. A white absorbent paper was cut to the size and shape of the dish and taped to the outer bottom of each dish to reduce glare from the lights and provide contrast for photographing. A section (approximately 14 cm) of each VX-contaminated leaf was cut from the

plant with a scissors at a point distal to the leaf tip and containing the VX spread. This section was placed inside the prepared Petri dish and secured with clear plastic tape at both ends. Leaf images were immediately digitally captured using a Canon EOS Digital Rebel XTi camera equipped with a Canon EF-S600 mm f /2.8 Macro USM lens (Canon USA, Inc.; Lake Success, NY). The camera was secured on a ringstand and leveled. The entire camera was covered with a clear plastic bag to protect it from contamination by VX. A hole was cut in the protective bag directly in front of the lens and then sealed with a clear skylight filter to prevent lens contamination. During each photography session, a Target Dot Diameter slide (Max Levy Autograph, Inc.; Philadelphia, PA) was used as a standardized calibration tool and digitally imaged to determine the SA of each VX droplet spread on each leaf. Multiple images of each leaf surface were saved to electronic files for analyses.

3.6 Quantification of VX Droplet Spread on the Leaf Surface

SA values of the VX droplet spreads were determined using Image-Pro Insight software, version 8.0 (Media Cybernetics, Inc.; Rockville, MD). Pixelation of the SA of the Target Dot was determined using the imaging software. The margins of each spread were traced with a mouse, and the number of pixels was counted, compared with the pixel count of the Target Dot, and converted to SA (square millimeters) by the software. Each image was traced and analyzed three times, and the average SA and standard error were used to estimate the SA of each droplet spread. The SA determination described herein was conducted for each of four leaf replicates per treatment (a treatment consisted of the time of exposure of the leaves to the CWA after dissemination). An analysis of variance and Fisher’s least-significant pairwise-comparison tests were used to determine if significant differences ($p < 0.05$) existed between mean SA values for each time period (SYSTAT 11.0; Systat Software, Chicago, IL).

4. RESULTS AND DISCUSSION

4.1 Visual Characterization of VX Injury on *E. crus-galli* Foliage

The visual appearances of the 1 and 3 µL droplet spreads of VX varied with time on the live plants. Upon initial contact with the leaf surface, the VX droplets were absorbed, which produced a wet “water-soaked” appearance of the leaf in affected areas. This wet appearance immediately spread outward from the initial location of the absorbed droplet in all directions, but favored migration parallel to the leaf veins and formed an oblong or oval pattern after 1 to 3 min and then continued spreading for up to 1 h (Figure 4a,b). The spread of VX droplets contrasted greatly from that of water droplets, which maintained a spherical shape, spread minimally if at all, and evaporated after 1 h (Figure 4c). After 1 h, the VX-affected area of the leaf surface appeared dark-colored and dry and apparently stopped spreading (Figure 5). At 4 h after dissemination of the VX droplets, the color of the affected area of the leaf transitioned from dark to tan, and the SA of the affected portion appeared to be shrinking (Figure 6). After 24 h of exposure to VX, the affected area appeared light tan and necrotic (dead tissue) (Figure 7). The spread of the injury was always finite in that the rest of the leaf remained green and healthy, and subsequent growth of the plant appeared to be unaffected.

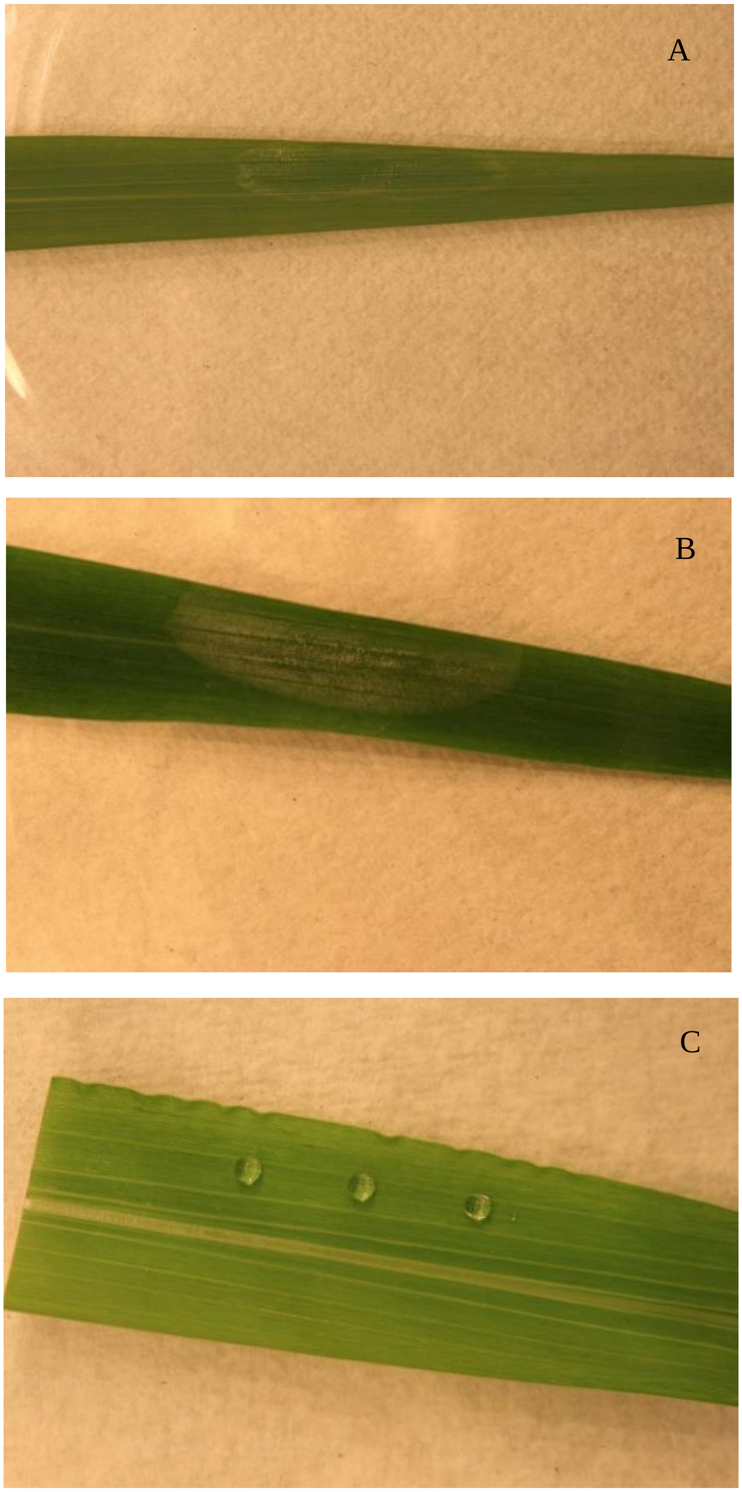

Figure 4. (A) Spread of one 1 µL VX droplet, (B) spread of one 3 µL VX droplet, and (C) spread of three 3 µL water droplets; each result was photographed on an *E. crus-galli* leaf at 0.05 h after dissemination onto the foliage.

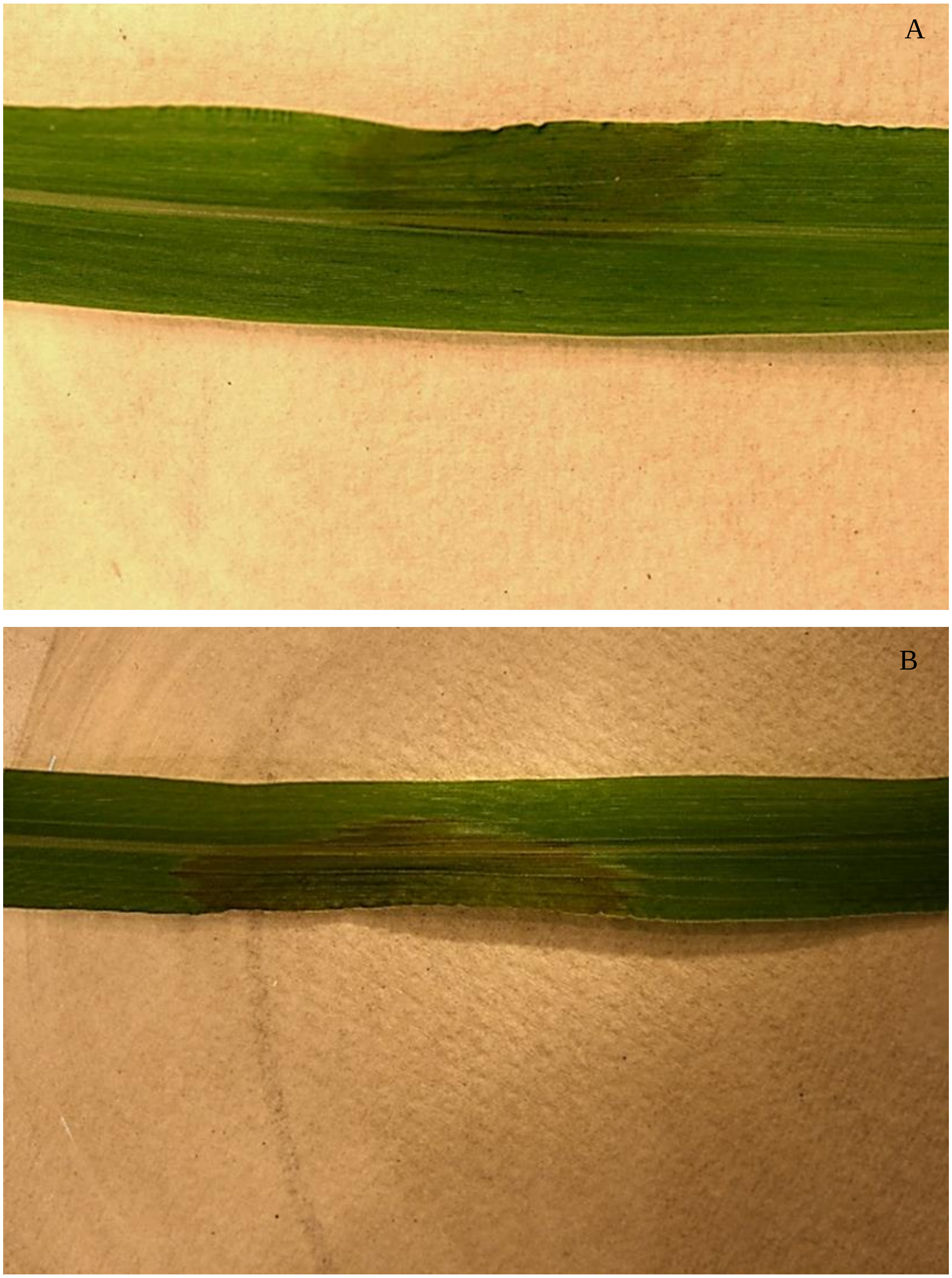

Figure 5. (A) Spread of one 1 µL VX droplet, and (B) spread of one 3 µL VX droplet; each result was photographed on an *E. crus-galli* leaf at 1 h after dissemination of VX onto the foliage.

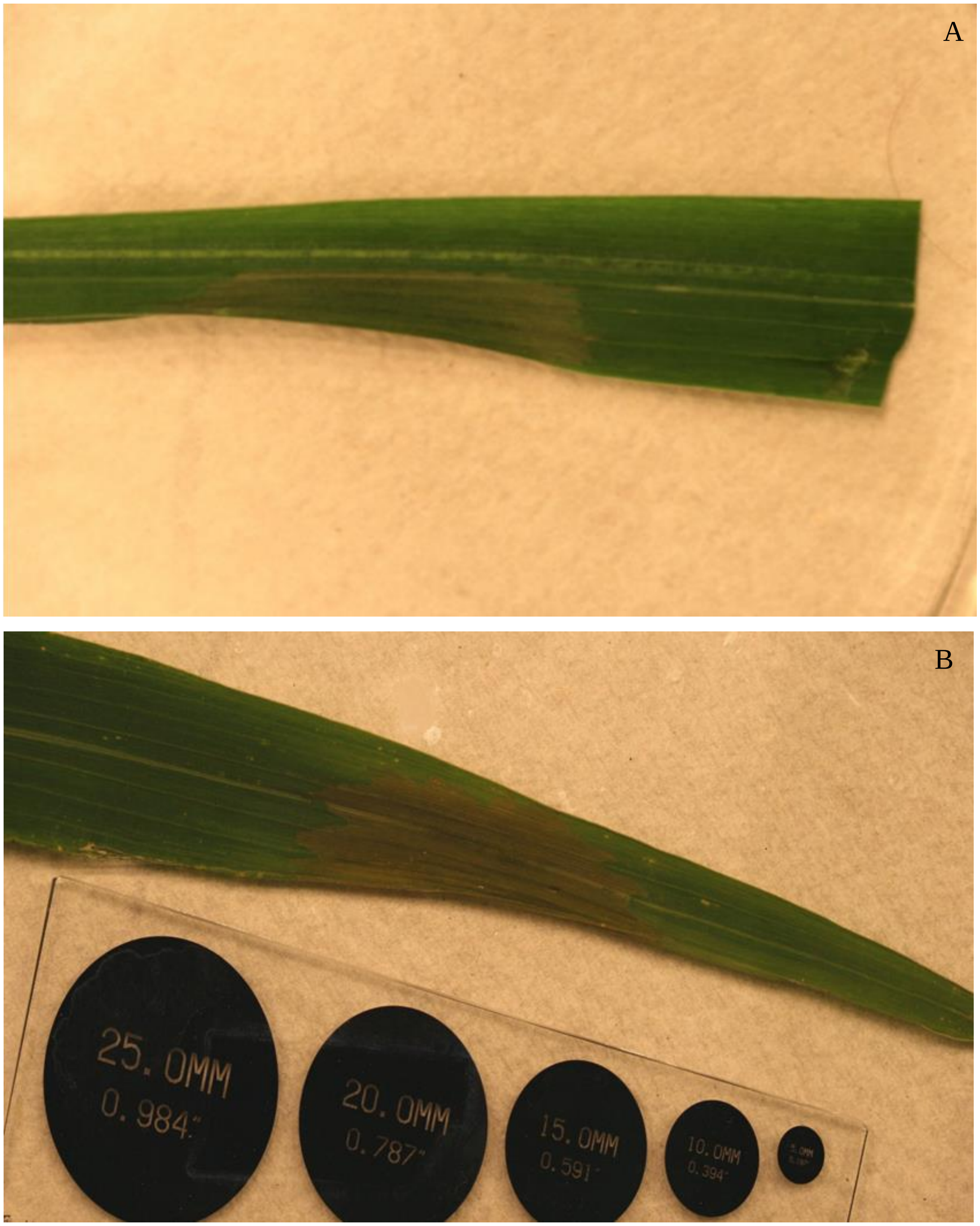

Figure 6. (A) Spread of one 1 µL VX droplet, and (B) Target Dot calibration discs and spread of one 3 µL VX droplet; each result was photographed on an *E. crus-galli* leaf at 4 h after dissemination of VX onto the foliage.

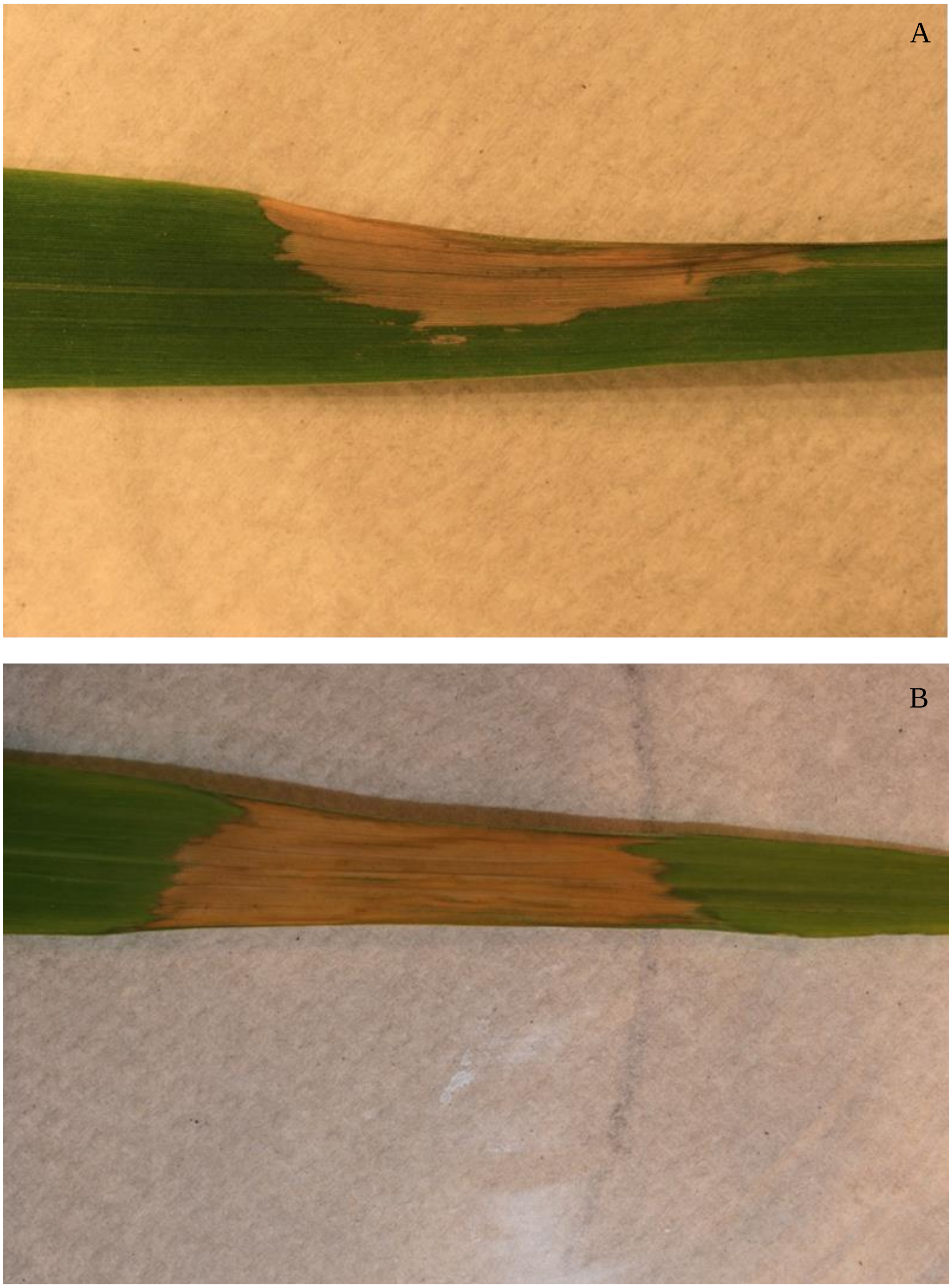

Figure 7. (A) Spread of one 1 µL VX droplet, and (B) spread of one 3 µL VX droplet; each result was photographed on an *E. crus-galli* leaf at 24 h after dissemination of VX onto the foliage.

The immediate VX droplet spread on leaf surfaces and absorption into foliage observed in this study indicated that VX is very miscible with the epicuticular waxes and cuticle on the leaf surface. Many factors affect the behavior, persistence, and fate of CWA droplets on leaf surfaces. When a liquid droplet rests on a solid surface, the contact angle is the controlling factor that determines the rate of evaporation or penetration into surfaces. The contact angle is determined by the surface tension of the droplet on a solid surface which, in turn, is determined by the chemical properties of the droplet and the surface (Gorzkowska-Sobas, 2013). Wettable surfaces, on which an applied drop of liquid tends to spread, have a low (<90°) contact angle (Koch and Barthlott, 2009). When the contact angle on a surface is 90° or greater, a water droplet forms a spherical shape, and the contact between the adhering droplet and the surface is very small (Koch and Barthlott, 2009). In this study, we did not directly measure the contact angle of the water or VX on the leaves. However, it was evident upon visual examination of the images that the resulting contact angle for water on the surface of *E. crus-galli* approached 90°, whereas the contact angle for VX was very low. The water droplets formed spherical shapes, did not spread, and evaporated completely after 1 h. The VX droplets spread immediately in all directions, but more so in the direction of the veins, forming an oval pattern, quickly absorbing into the leaf, and appearing fully absorbed after 1 h.

The majority of the literature describing CWA persistence and absorption on solid surfaces describes studies with nonporous and inanimate porous materials such as steel, glass, concrete, plastics, sand, or milled wood (D'Onofrio et al., 2010; Gorzkowska-Sobas, 2013; Groenewold et al., 2000; MacGregor et al., 2008; Wagner et al., 2001) or with living animal skin (D'Onofrio, 2013). Leaf surfaces of living plants are much different and unique. The outermost leaf surface is composed of epicuticular waxes, which are complex lipophilic mixtures of primarily long-chain aliphatics, including primary alcohols (*n*-alkan-1-ols), aldehydes, fatty acids, alkyl esters, and hydrocarbons and secondary alcohols and ketones (Walton, 1990). Beneath the epicuticular wax is the cuticle, which contains the hydrophilic cutin along with imbedded waxes (Ashton and Crafts, 1981). Therefore, the contact angle for a droplet on a leaf will be determined by the physical and chemical properties of both the liquid droplet and the epicuticular waxes of the leaf. Hence, the extent of spread, penetration, and evaporation of CWA droplets on leaf surfaces will depend on the respective hydrophilicity of the CWA and the hydrophobicity of the leaf surface. Based on the behavior of VX on leaf surfaces in the present study, the grass leaf surface and VX have properties that allow immediate absorption of VX into the leaf. The visual symptoms of VX damage on *E. crus-galli* leaves are functions of the physical and chemical properties of the VX, the leaf surface, and the internal plant tissue inter- and intracellular compositions. Using a CWA that is substantially more hydrophilic than VX will result in a droplet with a definitive contact angle on an *E. crus-galli* leaf. Likewise, contaminating a different plant species with leaf surface properties that are less similar to the chemical properties of VX will also result in a droplet with a definitive contact angle.

The limited spread and finite behavior of the droplets in this study may be explained by recent studies with *E. crus-galli* and primisulfuron, which is an herbicide. Scanning electron microscopic examination has shown that the leaf surface of *E. crus-galli* is covered with bicellular trichomes (leaf hairs) (Sanyal et al., 2006). Trichomes act in a complex way relative to spread of herbicide solution and sorption of herbicide. Trichomes may cause reduced wetting and spreading of aqueous droplets, which implies that the presence of trichomes would increase

wetting and spreading of more nonpolar liquids, such as VX. Adding an organosilicone wetting agent with primisulfuron increased the spread of the herbicide so that it covered more SA on leaves than had occurred without the wetting agent (Sanyal et al., 2006).

Intact living plants also respond metabolically to intrusion by chemical stressors. Bicellular trichomes discharge a mucilage-type secretion that contains callose, a carbohydrate component (1,3-glucan) usually associated with “walling off responses”, similar to those associated with injured plant tissues (McWhorter et al., 1995).

4.2 SAs of VX Droplet Spreads on Leaf Surfaces

Mean SA values of VX droplet spreads at various times after VX dissemination are shown in Figure 8. The mean SAs of the affected tissues for the 1 µL droplets were 132, 192, 163, 135, and 142 mm^2 at 0.017, 0.05, 1, 4, and 24 h, respectively, and those for the 3 µL droplets were 166, 301, 303, 278, and 274 mm^2 at 0.05, 1, 4, 24, and 48 h, respectively. The SAs of the 1 µL droplet spreads at both 0.05 and 1 h were significantly greater ($p < 0.05$) than the SA of the droplet spread at 0.017 h. The SAs of the 4 and 24 h 1 µL droplet spreads were less and were not significantly different ($p > 0.05$) from the 0.017 h droplet spread. The SA of the 3 µL droplet spread at 0.05 h was significantly less ($p < 0.05$) than the SAs of the droplet spreads at 1, 4, 24, and 48 h, but the SAs at these later times were not significantly different from each other. As noted in Section 4.1, the area affected by the spread of VX was necrotic after 24 h. We surmised that loss of fluids (including water) that resulted from cellular injury and cell necrosis reduced the SA of the subsequent droplet spread.

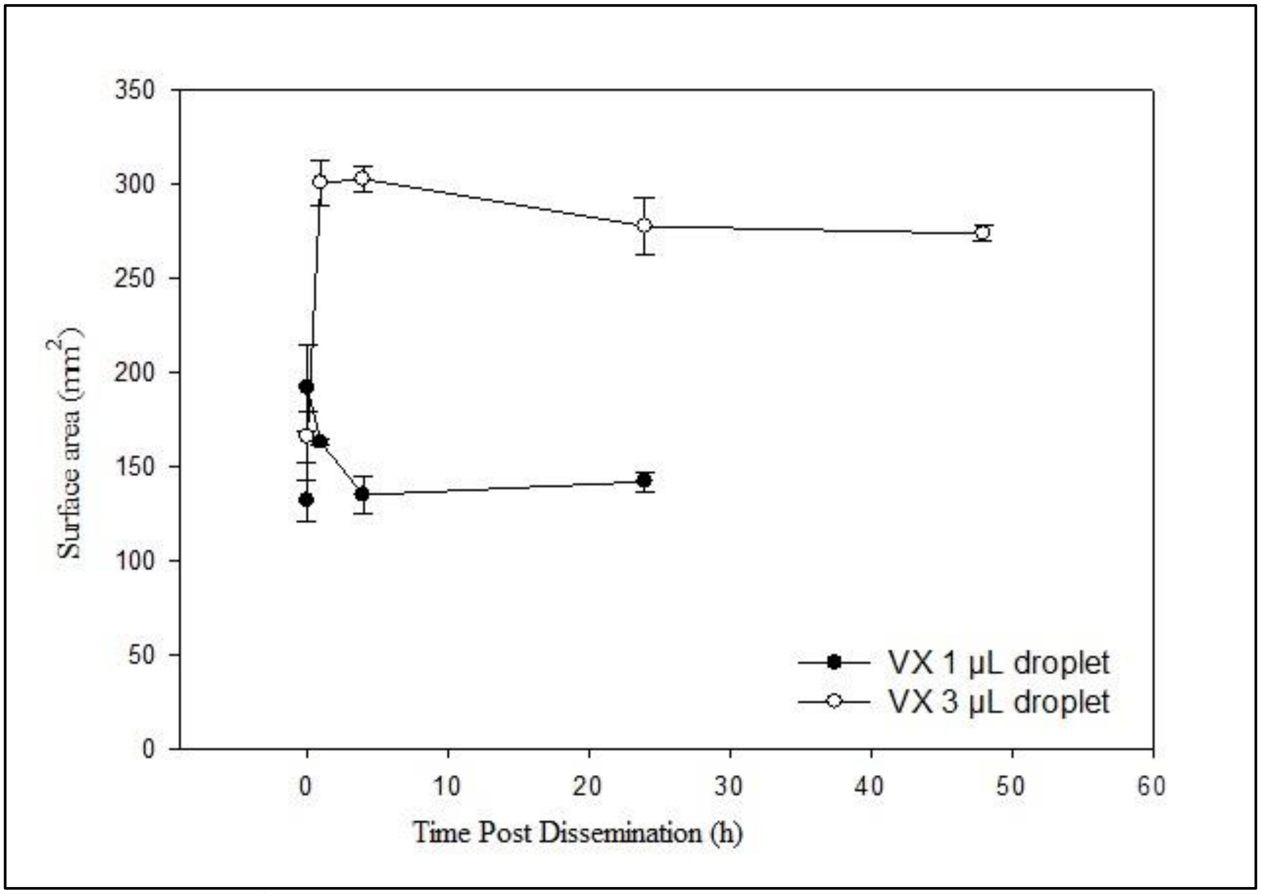

Figure 8. SAs (mm^2) of 1 and 3 µL VX droplet spreads on *E. crus-galli* leaves (n = 4, with three imaging determinations per replicate). Each error bar represents the standard error of the mean of four leaves.

4.3 Identification of Visual Leaf Injury Caused by an OP CWA

If a vegetated field is suspected of CWA contamination via aerial deposition, it is helpful to be able to identify the visual characteristics of CWA-induced injuries to foliage as compared with those from other possible causes such as insect infestation and symptoms of fungal, viral, and bacterial diseases. The characteristics of leaf damage due to the presence of CWA are similar to those caused by abiotic factors described by Green et al. (1990). Abiotic factors are nonliving entities such as pesticides, nutrient deficiencies or toxicities, or environmental conditions. On vegetation, symptoms that are caused by abiotic factors often affect several species or plants of various ages; typically, damage is relatively uniform, does not spread across fields, and is often not progressive. Abiotic-induced problems are not associated with pests; they are often caused by a single incident. Once the responsible factor has dissipated and is no longer affecting the plant, the plant can often grow out of the injury and develop new normal-appearing foliage (Tjovosvold and Koike, 2015). Tjovosvold and Koike also noted that injury caused by aerially applied chemicals may be identified by a band free from injury on a lower leaf, where leaves cross one another. The causal entity can be confirmed only by chemical

analyses of the affected plant tissues. However, the occurrence of such visual injuries on foliage, especially when CWA use is suspected within the region, can merit the use of standard battlefield detectors in symptomatic fields. Accelerated identification and characterization of fields suspected of CWA contamination can enhance the protection of Warfighters on the battlefield or that of first responders to events involving CWA use by terrorists.

Abiotic entities that are similar to OP CWAs, such as OP pesticides, may cause symptoms that are similar to those caused by OP CWAs. Methyl parathion, which is an OP insecticide, caused damage on leaves of susceptible sorghum varieties that was characterized by irregular or circular water-soaked necrotic blotches that tended to follow the leaf vein pattern (Edmunds and Zummo, 1975). The surface of the affected areas appeared to be dry after 1 h, indicating absorption of the methyl parathion into the interior leaf tissues. The spots dried out and turned necrotic within 72 h. The authors observed that sorghum plants generally outgrew the injury, and new growth was not affected. We observed a similar response from *E. crus-galli* grass leaves exposed to VX. The injury first appeared as water-soaked spots and progressed into oval-shaped patterns. The spots initially expanded, but stopped spreading after approximately 15 min. After 24 h, the spots became necrotic areas that were always finite and followed the leaf veins, with a clear demarcation between the damaged and healthy leaf tissues. Subsequent grass plant growth was not affected. Knowledge of site history, especially the recent application of pesticides on or near the vegetation in question, will also aid in the assessment and diagnosis of foliar injury that may be caused by VX.

In contrast, symptoms caused by biotic or living entities often affect one species or cultivar of the same age and are typically observed to be in random or irregular locations. These are indicators that help distinguish between abiotic (chemical) and biotic symptoms. Unlike abiotic effects, such biotic symptoms appear at varying times, and severity varies among affected plants. Biotic problems are infections with bacteria, viruses, spores, fungal mycelia, and other pathogenic entities that increase in distribution when environmental conditions are favorable for disease development and may be associated with pests that have affected a crop. This infectious aspect is important, as biotic diseases can be progressive and continue to affect additional tissues and more plants over time (Tjovosvold and Koike, 2015).

5. CONCLUSIONS

We successfully developed methods to study the spread of 1 and 3 µL VX droplets and to characterize the injury pattern over time on the leaf surfaces of living intact grass plants (*E. crus-galli*) growing in chemical surety hoods. Our study is the first to quantitatively characterize CWA droplets on leaf surfaces. After dissemination onto plant foliage, VX droplets immediately spread, forming a wet, water-soaked oblong pattern that followed the leaf veins. The droplet spread was finite and ceased to advance within 1 h. The visual pattern progressed from the initial water-soaked appearance to dark-colored, dark tan, and light tan (necrotic) within 1, 4, and 24 h after dissemination, respectively. The VX appeared to absorb into the leaf tissues. Quantification of the droplet spread images resulted in a greater SA from the 3 µL droplet spread compared with the 1 µL droplet spread. Information derived from these studies can accelerate the identification and characterization of fields suspected of being contaminated with CWAs and can

enhance the protection of Warfighters on the battlefield or first responders to terrorist events involving CWA. The information in this report can also aid efforts by the Department of Homeland Security, Environmental Protection Agency, and others involved with identifying and mitigating the environmental release of CWAs.

6. ONGOING AND FUTURE RELATED STUDIES

Ongoing related studies address the persistence, fate, and contact transfer of VX droplets on foliage. Additional critical parameters under investigation include the wash-off coefficient from measured rainfall, distribution of CWAs on and within leaves as a function of time, and contact transfer (exposure) of CWAs from contaminated foliar surfaces onto an army combat uniform. At the time of this report, analytical determinations of VX on foliar surfaces and within leaf tissues at the respective time points were in progress. Results from the analytical determinations will be used to determine the effective half-life of VX on grass foliage. Effective half-life is a measurement of the net effect of all factors affecting CWA persistence including evaporation, transformation, and fixation. Droplet spread SAs derived in this study will be used in the determination of wash-off coefficients for CWA fate models, based on the water volume of simulated rain and SA values for droplet spread. Results from these investigations will provide critical parameter input for predictive models, direct experimental determinations for comparison of predictive model outcomes, and information for decision-making that will affect Soldiers on CWA-contaminated battlefields.

LITERATURE CITED

Ashton, F.M.; Crafts, A.S. *Mode of Action of Herbicides*, 2nd ed.; John Wiley & Sons: New York, 1981.

ASTM International. *Standard Specification for Reagent Water*; ASTM D1193-99; ASTM International: West Conshohocken, PA, 2004; UNCLASSIFIED Specification. http://www.astm.org/DATABASE.CART/HISTORICAL/D1193-99.htm (accessed December 2015).

CABI. *Echinochloa crus-galli.* In *Crop Protection Compendium*. CAB International: Wallingford, UK. http://www.cabi.org/cpc (accessed January 2016).

Chemical and Biological Contamination Survivability (CBCS), Large Item Exteriors; Test Operations Procedure (TOP) 08-2-510A; U.S. Army Dugway Proving Ground, West Desert Test Center: Dugway, UT, 2011; UNCLASSIFIED Report.

D'Onofrio, T.G. *Development of a Contact Permeation Test Fixture and Method*; ECBC-TR-1141; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2013. UNCLASSIFIED Report (ADA583691).

D'Onofrio, T.G.; Navaz, H.K.; Markicevic, B.; Mantooth, B.A.; Sumpter, K.B. Experimental and Numerical Study of Spread and Sorption of VX Sessile Droplets into Medium Grain-Size Sand. *Langmuir* **2010,** *26* (5), 3317–3322.

Edmunds, L.K.; Zummo, N. *Sorghum Diseases in the United States and Their Control*; Agriculture Handbook No. 468; Agricultural Research Service, U.S. Department of Agriculture: Washington, DC, 1975.

Gorzkowska-Sobas, A.A. *Chemical Warfare Agents and Their Interactions with Solid Surfaces*; FFI-rapport 2013/00574; Norwegian Defence Research Establishment (FFI): Kjeller, Norway, 2013.

Green, J.L.; Capizi, J.; Maloy, O.A. *A Systematic Approach to Diagnosing Plant Damage*. Ornamentals Northwest Archives, Oregon State University: Corvallis, OR, 1990; Vol. 13, pp 1–37.

Groenewold, G.S.; Appelhans, A.D.; Gresham, G.L.; Olson, J.E.; Jeffery, M.; Weibel, M. Characterization of VX on Concrete Using Ion Trap Secondary Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2000,** *11*, 69–77.

Koch, K.; Barthlott, W. Superhydrophobic and Superhydrophilic Plant Surfaces: An Inspiration for Biomimetic Materials. *Phil. Trans. A Math Phys. Eng. Sci.* **2009,** *367*, 1487–1509.

MacGregor, I.C.; Rogers, J.V.; Kenny, D.V.; Hayes, T.; Taylor, M.L.; Nishioka, M.G.; Riggs, K.B.; Willenberg, Z.J.; Krile, R.T.; Stone, H.J. *Persistence of Toxic Industrial Chemicals and Chemical Warfare Agents on Building Materials under Conventional Environmental Conditions*; EPA/600/R-08/075; National Homeland Security Research Center, U.S. Environmental Protection Agency: Research Triangle Park, NC, 2008.

McCree, K.J. Photosynthetically Active Radiation. In *Physiological Plant Ecology I*, Encyclopedia of Plant Physiology, Vol. 12A; Springer-Verlag: Berlin, 1981; pp 41–55.

McWhorter, C.G.; Paul, R.N.; Ouzts, J.C. Bicellular Trichomes of Johnsongrass (*Sorghum halepense*) Leaves: Morphology, Histochemistry, and Function. *Weed Science* **1995,** *43* (2), 201–208.

Munro, N.B.; Ambrose, K.R.; Watson, A.P. Toxicity of the Organophosphate Chemical Warfare Agents GA, GB, and VX: Implications for Public Protection. *Environ. Health Perspect*. **1994,** *102*, 18–38.

Reutter, S.A. Hazards of Chemical Weapons Released during War: New Perspectives. *Environ. Health Perspect.* **1999,** *107* (12), 985–990.

Sanyal, D.; Bhowmik, P.C.; Prasanta, C.; Reddy, K.N. Influence of Leaf Surface Micromorphology, Wax Content, and Surfactant on Primisulfuron Droplet Spread on Barnyardgrass (*Echinochloa crus-galli*) and Green Foxtail (*Setaria viridis*). *Weed Science* **2006,** *54* (4), 627–633.

Tjovosvold, S.; Koike, S. Distinguishing Abiotic and Biotic Symptoms in Plants, Topics in Subtropics; University of California; Posted 29 May 2015. http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=17827 (accessed January 2016).

U.S. Department of Agriculture Natural Resource Conservation Service (USDA NRCS), The PLANTS Database; National Plant Data Team: Greensboro, NC, 2016. http://plants.usda.gov (accessed January 2016).

Van Kampen, K.R.; James, L.F.; Rasmussen, J.; Huffaker, R.H.; Fawcett, M.O. Organic Phosphate Poisoning in Sheep in Skull Valley, Utah. *J. Am. Med. Assoc.* **1969,** *154*, 623–630.

Van Kampen, K.R.; Shupe, J.L.; Johnson, A.E.; James, L.F.; Smart, R.A.; Rasmussen, J.E. Effects of Nerve Gas Poisoning in Sheep in Skull Valley, Utah. *J. Am. Med. Assoc.* **1970,** *156*, 1032–1035.

Wagner, G.W.; O'Conner, R.J.; Procell, L.R. Preliminary Study on the Fate of VX in Concrete. *Langmuir* **2001,** *17* (14), 4336–4341.

Walton, T.J. Waxes, Cutin, and Suberin. In *Methods in Plant Biochemistry*; Vol. 4; Dey, P.M., Harborne, J.B., Eds.; Academic Press: San Diego, CA, 1990; pp 105–158.

Watson, A.P.; Ambrose, K.R.; Griffin, G.D.; Leffingwell, S.S.; Munro, N.B.; Waters, L.C. Health Effects of Warfare Agent Exposure: Implications for Stockpile Disposal. *Environ. Professional* **1989,** *11*, 335–353.

Blank

ACRONYMS AND ABBREVIATIONS

CAS	Chemical Abstracts Service
CWA	chemical warfare agent
LED	light-emitting diode
OP	organophosphorus
p	probability
PAR	photosynthetically active radiation
RH	relative humidity
SA	surface area
VX	*O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothioate

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical
Biological Center (ECBC)
Molecular Toxicology Branch
RDCB-DRT-M
ATTN: Simini, M.
Checkai, R.
Haley, M.
Kristovich, R.
Whalley, C.

ECBC Chemical Sciences Division
RDCB-DRC
ATTN: Berg, F.

ECBC Agent Chemistry Branch
RDCB-DRC-C
ATTN: King, B.

Defense Threat Reduction Agency
J9-CBS
ATTN: Moore, E.
Minyard, M.

Department of Homeland Security
DHS-ORD-CSAC
ATTN: Famini, G.

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

Defense Technical Information Center
ATTN: DTIC OA

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.

EDGEWOOD CHEMICAL BIOLOGICAL CENTER

A U.S. Army RDECOM Laboratory